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(54) Title: METALLOPROTEINASE INHIBITOR SEQUENCE RECOMBINANT VECTOR SYSTEM FOR USING SAME AND RECOMBINANT-DNA METHOD FOR THE MANUFACTURE OF SAME

(57) Abstract

A portable DNA sequence which is capable of directing intracellular production of metalloproteinase inhibitors. Vectors containing this portable DNA sequence are also set forth, including the vector pUC9-F5/237P10. A recombinant-DNA method for the microbial production of a metalloproteinase inhibitor, which method incorporates at least one of the portable DNA sequences and the vectors disclosed herein.

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METALLOPROTEINASE INHIBITOR SEQUENCE RECOMBINANT VECTOR SYSTEM FOR USING SAME AND RECOMBINANT-DNA METHOD FOR THE MANUFACTURE OF SAME

BACKGROUND OF THE INVENTION

This is a continuation-in-part application of U.S. Patent Application Serial No. 784,319, filed October 4, 1985, which is a continuation-in-part application of U.S. Patent Application Serial No. 699,181, filed February 5, 1985. Endogenous proteolytic enzymes serve to degrade invading organisms, antigen-antibody complexes and certain tissue proteins which are no longer necessary or useful to the organism. In a normally functioning organism, proteolytic enzymes are produced in a limited quantity and are regulated in part through specific inhibitors.

Metalloproteinases are enzymes present in the body which are often involved in the degradation of connective tissue. While some connective tissue degradation is necessary for normal functioning of an organism, an excess of connective tissue degradation occurs in several disease states and is believed to be attributable, at least in part, to excess metalloproteinase. It is believed that metalloproteinases are at least implicated in periodontal disease, corneal and skin ulcers, rheumatoid arthritis and the spread of cancerous solid tumors.

These diseases generally occur in areas of the body which contain a high proportion of collagen, a particular form of connective tissue. An examination of patients with these diseases of connective tissue has revealed an excessive breakdown of the various components of connective tissues, including collagen proteoglycans and elastin. Therefore, it has been deduced that an excessive concentration of a particular metalloproteinase, for example collagenase, proteoglyconase, gelatinase, and certain elastases, may cause or exacerbate the connective tissue destruction associated with the aforementioned diseases.

In the normal state, the body possesses metalloproteinase inhibitors which bind to metalloproteinases to effectively prevent these enzymes from acting on their connective tissue substrates. Specifically, in a healthy organism, metalloproteinase inhibitors are present in concentrations sufficient to interact with metalloproteinases to an extent which allows sufficient quantities of metalloproteinase to remain active while binding the excess metalloproteinase so that the

connective tissue damage seen in the various diseases does not occur.

It is postulated that one immediate cause of the connective tissue destruction present in the foregoing disease states is an imbalance in the relative metalloproteinase/metalloproteinase inhibitor concentrations. In these situations, either due to an excessive amount of active metalloproteinase or a deficiency in the amount of active metalloproteinase inhibitor, the excess metalloproteinase is believed to cause the connective tissue degradation responsible for causing or exacerbating the disease. It is postulated that, by treating persons with connective tissue diseases with metalloproteinase inhibitors, the degradative action of the excess metalloproteinase may be curtailed or halted. Therefore, particular metalloproteinase inhibitors of specific interest to the present inventors are collagenase inhibitors because it is believed that these inhibitors would be pharmaceutically useful in the treatment or prevention of connective tissue diseases.

The existence of metalloproteinase and metalloproteinase inhibitors has been discussed in the scientific literature. For example, Sellers et al., Biochemical And Biophysical Research Communications 87:581-587 (1979), discusses isolation of rabbit bone collagenase inhibitor. Collagenase inhibitor isolated from human skin fibroblasts is discussed in Stricklin and Welgus, J.B.C. 258:12252-12258 (1983) and Welgus and Stricklin, J.B.C. 258:12259-12264 (1983). The presence of collagenase inhibitors in naturally-occurring body fluids is further discussed in Murphy et al., Biochem. J. 195:167-170 (1981) and Cawston et al., Arthritis and Rheumatism, 27:285 (1984). In addition, metalloproteinase inhibitors are discussed by Reynolds et al. in Cellular Interactions, Dingle and Gordon, eds., (1981). Although these articles characterize particular, isolated metalloproteinase inhibitors and discuss, to some extent, the role or potential role of metalloproteinases in connective tissue disease treatment and speculate on the ability of metalloproteinase inhibitors to counteract this destruction, none of these researchers had previously been able to isolate a portable DNA

sequence capable of directing intracellular production of metalloproteinase inhibitors or to create a recombinant-DNA method for the production of these inhibitors.

Surprisingly, the present inventors have discovered a portable DNA sequence capable of directing the recombinant-DNA synthesis of metalloproteinase inhibitors. These metalloproteinase inhibitors are biologically equivalent to those isolated from human skin fibroblast cultures. The metalloproteinase inhibitors of the present invention, prepared by the recombinant-DNA methods set forth herein, will enable increased research into prevention and treatment of metalloproteinase-induced connective tissue diseases. In addition, the metalloproteinase inhibitors of the present invention are useful in neutralizing metalloproteinases, including the excess metalloproteinase associated with disease states. Therefore, it is believed that a cure for these diseases will be developed which will embody, as an active ingredient, the metalloproteinase inhibitors of the present invention. Furthermore, the metalloproteinase inhibitors of the present invention are capable of interacting with their metalloproteinase targets in a manner which allows the development of diagnostic tests for degradative connective tissue diseases using the newly discovered inhibitors.

The recombinant metalloproteinase inhibitors discussed herein interact stoichiometrically (i.e., in a 1:1 ratio) with their metalloproteinase targets. In addition, these metalloproteinase inhibitors are heat resistant, acid stable, glycosylated, and exhibit a high isoelectric point.

SUMMARY OF THE INVENTION

The present invention relates to metalloproteinase inhibitors and a recombinant-DNA method of producing the same and to portable DNA sequences capable of directing intracellular production of the metalloproteinase inhibitors. Particularly, the present invention relates to a collagenase inhibitor, a recombinant-DNA method for producing the same and to portable DNA sequences for use in the recombinant method. The present invention also relates to a series of vectors containing these portable DNA sequences.

One object of the present invention is to provide a metalloproteinase inhibitor, which can be produced in sufficient quantities and purities to provide economical pharmaceutical compositions which possess metalloproteinase inhibitor activity.

An additional object of the present invention is to provide a recombinant-DNA method for the production of these metalloproteinase inhibitors. The recombinant metalloproteinase inhibitors produced by this method are biologically equivalent to the metalloproteinase inhibitor isolable from human skin fibroblast cultures.

To facilitate the recombinant-DNA synthesis of these metalloproteinase inhibitors, it is a further object of the present invention to provide portable DNA sequences capable of directing intracellular production of metalloproteinase inhibitors. It is also an object of the present invention to provide cloning vectors containing these portable sequences. These vectors are capable of being used in recombinant systems to produce pharmaceutically useful quantities of metalloproteinase inhibitors.

Additional objects and advantages of the invention will be set forth in part in the description which follows, and in part will be obvious from the description or may be learned from practice of the invention. The objects and advantages may be realized and attained by means of the instrumentalities and combinations particularly pointed out in the appended claims.

To achieve the objects and in accordance with the purposes of the present invention, metalloproteinase inhibitors are set forth, which are capable of stoichiometric reaction with metalloproteinases. These metalloproteinase inhibitors are remarkably heat resistant, acid stable, glycosylated, and exhibit a high isoelectric point. Furthermore, these metalloproteinase inhibitors are biologically equivalent to those inhibitors isolated from human skin fibroblast cultures.

To further achieve the objects and in accordance with the purposes of the present invention, as embodied and broadly described herein, portable DNA sequences coding for metalloproteinase inhibitors are provided. These sequences comprise

nucleotide sequences capable of directing intracellular production of metalloproteinase inhibitors. The portable sequences may be either synthetic sequences or restriction fragments ("natural" DNA sequences). In a preferred embodiment, a portable DNA sequence is isolated from a human fibroblast cDNA library and is capable of directing intracellular production of a collagenase inhibitor which is biologically equivalent to that inhibitor which is isolable from a human skin fibroblast culture.

The coding strand of a first preferred DNA sequence which has been discovered has the following nucleotide sequence:

```
        10          20          30          40          50          60
GTTGTTGCTG TGGCTGATAG CCCCAGCAGG GCCTGCACCT GTGTCCCACC CCACCCACAG

        70          80          90          100         110         120
ACGGCCTTCT GCAATTCCGA CCTCGTCATC AGGGCCAAGT TCGTGGGGAC ACCAGAAGTC

       130         140         150         160         170         180
AACCAGACCA CCTTATACCA GCGTTATGAG ATCAAGATGA CCAAGATGTA TAAAGGGTTC

       190         200         210         220         230         240
CAAGCCTTAG GGGATGCCGC TGACATCCGG TTCGTCTACA CCCCCGCCAT GGAGAGTGTC

       250         260         270         280         290         300
TGCGGATACT TCCACAGGTC CCACAACCGC AGCGAGGAGT TTCTCATTGC TGGAAAACTG

       310         320         330         340         350         360
CAGGATGGAC TCTTGCACAT CACTACCTGC AGTTTCGTGG CTCCCTGGAA CAGCCTGAGC

       370         380         390         400         410         420
TTAGCTCAGC GCCGGGGCTT CACCAAGACC TACACTGTTG GCTGTGAGGA ATGCACAGTG

       430         440         450         460         470         480
TTTCCCTGTT TATCCATCCC CTGCAAACTG CAGAGTGGCA CTCATTGCTT GTGGACGGAC

       490         500         510         520         530         540
CAGCTCCTCC AAGGCTCTGA AAAGGGCTTC CAGTCCCGTC ACCTTGCCTG CCTGCCTCGG
```

```
      550         560         570         580         590         600
GAGCCAGGGC TGTGCACCTG GCAGTCCCTG CGGTCCCAGA TAGCCTGAAT CCTGCCCGGA

      610         620         630         640         650         660
GTGGAAGCTG AAGCCTGCAC AGTGTCCACC CTGTTCCCAC TCCCATCTTT CTTCCGGACA

      670         680         690         700
ATGAAATAAA GAGTTACCAC CCAGCAAAAA AAAAAAGGAA TTC
```

The nucleotides represented by the foregoing abbreviations are set forth in the Detailed Description of the Preferred Embodiments.

A second preferrred DNA sequence has been discovered which has an additional nucleotide sequence 5' to the initiator sequence. This sequence, which contains as the eighty-second through four-hundred-thirty-second nucleotides nucleotoides 1 through 351 of the first preferred sequence set forth above, has the following nucleotide sequence:

```
       10          20          30          40          50          60
GGCCATCGCC GCAGATCCAG CGCCCAGAGA GACACCAGAG AACCCACCAT GGCCCCCTTT

       70          80          90         100         110         120
GACCCCTGGC TTCTGCATCC TGTTGTTGCT GTGGCTGATA GCCCCAGCAG GGCCTGCACC

      130         140         150         160         170         180
TGTGTCCCAC CCCACCCACA GACGGCCTTC TGCAATTCCG ACCTCGTCAT CAGGGCCAAG

      190         200         210         220         230         240
TTCGTGGGGA CACCAGAAGT CAACCAGACC ACCTTATACC AGCGTTATGA GATCAAGATG

      250         260         270         280         290         300
ACCAAGATGT ATAAAGGGTT CCAAGCCTTA GGGGATGCCG CTGACATCCG GTTCGTCTAC

      310         320         330         340         350         360
ACCCCCGCCA TGGAGAGTGT CTGCGGATAC TTCCACAGGT CCCACAACCG CAGCGAGGAG

      370         380         390         400         410         420
TTTCTCATTG CTGGAAAACT GCAGGATGGA CTCTTGCACA TCACTACCTG CAGTTTCGTG
```

430
GCTCCCTGGA AC

A third preferred DNA sequence which incorporates the 5' region of the second preferred sequence and the 3' sequence of the first preferred sequence, has the following nucleotide sequence:

```
        10          20          30          40          50          60
GGCCATCGCC GCAGATCCAG CGCCCAGAGA GACACCAGAG AACCCACCAT GGCCCCCTTT

        70          80          90          100         110         120
GACCCCTGGC TTCTGCATCC TGTTGTTGCT GTGGCTGATA GCCCCAGCAG GGCCTGCACC

        130         140         150         160         170         180
TGTGTCCCAC CCCACCCACA GACGGCCTTC TGCAATTCCG ACCTCGTCAT CAGGGCCAAG

        190         200         210         220         230         240
TTCGTGGGGA CACCAGAAGT CAACCAGACC ACCTTATACC AGCGTTATGA GATCAAGATG

        250         260         270         280         290         300
ACCAAGATGT ATAAAGGGTT CCAAGCCTTA GGGGATGCCG CTGACATCCG GTTCGTCTAC

        310         320         330         340         350         360
ACCCCCGCCA TGGAGAGTGT CTGCGGATAC TTCCACAGGT CCCACAACCG CAGCGAGGAG

        370         380         390         400         410         420
TTTCTCATTG CTGGAAAACT GCAGGATGGA CTCTTGCACA TCACTACCTG CAGTTTCGTG

        430         440         450         460         470         480
GCTCCCTGGA ACAGCCTGAG CTTAGCTCAG CGCCGGGGCT TCACCAAGAC CTACACTGTT

        490         500         510         520         530         540
GGCTGTGAGG AATGCACAGT GTTTCCCTGT TTATCCATCC CCTGCAAACT GCAGAGTGGC

        550         560         570         580         590         600
ACTCATTGCT TGTGGACGGA CCAGCTCCTC CAAGGCTCTG AAAAGGGCTT CCAGTCCCGT
```

610 620 630 640 650 660
CACCTTGCCT GCCTGCCTCG GGAGCCAGGG CTGTGCACCT GGCAGTCCCT GCGGTCCCAG

670 680 690 700 710 720
ATAGCCTGAA TCCTGCCCGG AGTGGAAGCT GAAGCCTGCA CAGTGTCCAC CCTGTTCCCA

730 740 750 760 770 780
CTCCCATCTT TCTTCCGGAC AATGAAATAA AGAGTTACCA CCCAGCAAAA AAAAAAAGGA

Currently, for expression of the instant metalloproteinase inhibitors in animal cells, the inventors most prefer a method which utilizes a fourth preferred DNA sequence. The coding strand of this sequence reads as follows:

10 20 30 40 50 60
GGCCATCGCC GCAGATCCAG CGCCCAGAGA GACACCAGAG AACCCACCAT GGCCCCCTTT

70 80 90 100 110 120
GAGCCCCTGG CTTCTGGCAT CCTGTTGTTG CTGTGGCTGA TAGCCCCCAG CAGGGCCTGC

130 140 150 160 170 180
ACCTGTGTCC CACCCCACCC ACAGACGGCC TTCTGCAATT CCGACCTCGT CATCAGGGCC

190 200 210 220 230 240
AAGTTCGTGG GGACACCAGA AGTCAACCAG ACCACCTTAT ACCAGCGTTA TGAGATCAAG

250 260 270 280 290 300
ATGACCAAGA TGTATAAAGG GTTCCAAGCC TTAGGGGATG CCGCTGACAT CCGGTTCGTC

310 320 330 340 350 360
TACACCCCCG CCATGGAGAG TGTCTGCGGA TACTTCCACA GGTCCCACAA CCGCAGCGAG

370 380 390 400 410 420
GAGTTTCTCA TTGCTGGAAA ACTGCAGGAT GGACTCTTGC ACATCACTAC CTGCAGTTTC

430 440 450 460 470 480
GTGGCTCCCT GGAACAGCCT GAGCTTAGCT CAGCGCCGGG GCTTCACCAA GACCTACACT

490 500 510 520 530 540
GTTGGCTGTG AGGAATGCAC AGTGTTTCCC TGTTTATCCA TCCCCTGCAA ACTGCAGAGT

```
      550         560         570         580         590         600
GGCACTCATT GCTTGTGGAC GGACCAGCTC CTCCAAGGCT CTGAAAAGGG CTTCCAGTCC

      610         620         630         640         650         660
CGTCACCTTG CCTGCCTGCC TCGGGAGCCA GGGCTGTGCA CCTGGCAGTC CCTGCGGTCC

      670         680         690         700         710         720
CAGATAGCCT GAATCCTGCC CGGAGTGGAA GCTGAAGCCT GCACAGTGTC CACCCTGTTC

      730         740         750         760         770         780
CCACTCCCAT CTTTCTTCCG GACAATGAAA TAAAGAGTTA CCACCCAGCA AAAAAAAAAA

GGAATTC
```

To facilitate identification and isolation of natural DNA sequences for use in the present invention, the inventors have developed a human skin fibroblast cDNA library. This library contains the genetic information capable of directing a cell to synthesize the metalloproteinase inhibitors of the present invention. Other natural DNA sequences which may be used in the recombinant DNA methods set forth herein may be isolated from human genomic libraries.

Additionally, portable DNA sequences useful in the processes of the present invention may be synthetically created. These synthetic DNA sequences may be prepared by polynucleotide synthesis and sequencing techniques known to those of ordinary skill in the art.

Additionally, to achieve the objects and in accordance with the purposes of the present invention, a recombinant-DNA method is disclosed which results in microbial manufacture of the instant metalloproteinase inhibitors using the portable DNA sequences referred to above. This recombinant DNA method comprises:

(a) preparation of a portable DNA sequence capable of directing a host microorganism to produce a protein having metalloproteinase inhibitor activity, preferably collagenase inhibitor activity;

(b) cloning the portable DNA sequence into a vector capable of being transferred into and replicating in a host microorganism, such vector containing operational elements for the portable DNA sequence;

(c) transferring the vector containing the portable DNA sequence and operational elements into a host microorganism capable of expressing the metalloproteinase inhibitor protein;

(d) culturing the host microorganism under conditions appropriate for amplification of the vector and expression of the inhibitor; and

(e) in either order:

(i) harvesting the inhibitor; and

(ii) causing the inhibitor to assume an active, tertiary structure whereby it possesses metalloproteinase inhibitor activity.

To further accomplish the objects and in further accord with the purposes of the present invention, a series of cloning vectors are provided comprising at least one of the portable DNA sequences discussed above. In particular, plasmid pUC9-F5/237P10 is disclosed.

It is understood that both the foregoing general description and the following detailed description are exemplary and explanatory only and are not restrictive of the invention, as claimed.

The accompanying drawing, which is incorporated in and constitutes a part of this specification, illustrates one embodiment of the invention and, together with the description, serves to explain the principles of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a partial restriction map of the plasmid pUC9-F5/237P10.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Reference will now be made in detail to the presently preferred embodiments of the invention, which, together with the drawing and the following examples, serve to explain the principles of the invention.

As noted above, the present invention relates in part to portable DNA sequences capable of directing intracellular production of metalloproteinase inhibitors in a variety of host microorganisms. "Portable DNA sequence" in this context is intended to refer either to a synthetically-produced nucleotide sequence or to a restriction fragment of a naturally occuring DNA sequence. For purposes of this specification, "metalloproteinase inhibitor" is intended to mean the primary structure of the protein as defined by the codons present in the deoxyribonucleic acid sequence which directs intracellular production of the amino acid sequence, and which may or may not include post-translational modifications. It is contemplated that such post-translational modifications include, for example, glycosylation. It is further intended that the term "metalloproteinase inhibitor" refers to either the form of the protein as would be excreted from a microorganism or the methionyl-metalloproteinase inhibitor as it may be present in microorganisms from which it was not excreted.

In a preferred embodiment, the portable DNA sequences are capable of directing intracellular production of collagenase inhibitors. In a particularly preferred embodiment, the portable DNA sequences are capable of directing intracellular production of a collagenase inhibitor biologically equivalent to that previously isolated from human skin fibroblast cultures. By "biologically equivalent," as used herein in the specification and claims, it is meant that an inhibitor, produced using a portable DNA sequence of the present invention, is capable of preventing collagenase-induced tissue damage of the same type, but not necessarily to the same degree, as a native human collagenase inhibitor, specifically that native human collagenase inhibitor isolable from human skin fibroblast cell cultures.

A first preferred portable DNA sequence of the present invention has a nucleotide sequence as follows:

```
        10          20          30          40          50          60
GTTGTTGCTG TGGCTGATAG CCCCAGCAGG GCCTGCACCT GTGTCCCACC CCACCCACAG

        70          80          90         100         110         120
ACGGCCTTCT GCAATTCCGA CCTCGTCATC AGGGCCAAGT TCGTGGGGAC ACCAGAAGTC

       130         140         150         160         170         180
AACCAGACCA CCTTATACCA GCGTTATGAG ATCAAGATGA CCAAGATGTA TAAAGGGTTC

       190         200         210         220         230         240
CAAGCCTTAG GGGATGCCGC TGACATCCGG TTCGTCTACA CCCCCGCCAT GGAGAGTGTC

       250         260         270         280         290         300
TGCGGATACT TCCACAGGTC CCACAACCGC AGCGAGGAGT TTCTCATTGC TGGAAAACTG

       310         320         330         340         350         360
CAGGATGGAC TCTTGCACAT CACTACCTGC AGTTTCGTGG CTCCCTGGAA CAGCCTGAGC

       370         380         390         400         410         420
TTAGCTCAGC GCCGGGGCTT CACCAAGACC TACACTGTTG GCTGTGAGGA ATGCACAGTG

       430         440         450         460         470         480
TTTCCCTGTT TATCCATCCC CTGCAAACTG CAGAGTGGCA CTCATTGCTT GTGGACGGAC

       490         500         510         520         530         540
CAGCTCCTCC AAGGCTCTGA AAAGGGCTTC CAGTCCCGTC ACCTTGCCTG CCTGCCTCGG

       550         560         570         580         590         600
GAGCCAGGGC TGTGCACCTG GCAGTCCCTG CGGTCCCAGA TAGCCTGAAT CCTGCCCGGA

       610         620         630         640         650         660
GTGGAAGCTG AAGCCTGCAC AGTGTCCACC CTGTTCCCAC TCCCATCTTT CTTCCGGACA

       670         680         690         700
ATGAAATAAA GAGTTACCAC CCAGCAAAAA AAAAAAGGAA TTC
```

wherein the following nucleotides are represented by the abbreviations indicated below.

Nucleotides	Abbreviation
Deoxyadenylic acid	A
Deoxyguanylic acid	G
Deoxycytidylic acid	C
Thymidylic acid	T

A second preferred portable DNA sequence of the present invention has the following nucleotide sequence:

```
        10          20          30          40          50          60
GGCCATCGCC GCAGATCCAG CGCCCAGAGA GACACCAGAG AACCCACCAT GGCCCCCTTT

        70          80          90         100         110         120
GACCCCTGGC TTCTGCATCC TGTTGTTGCT GTGGCTGATA GCCCCAGCAG GGCCTGCACC

       130         140         150         160         170         180
TGTGTCCCAC CCCACCCACA GACGGCCTTC TGCAATTCCG ACCTCGTCAT CAGGGCCAAG

       190         200         210         220         230         240
TTCGTGGGGA CACCAGAAGT CAACCAGACC ACCTTATACC AGCGTTATGA GATCAAGATG

       250         260         270         280         290         300
ACCAAGATGT ATAAAGGGTT CCAAGCCTTA GGGGATGCCG CTGACATCCG GTTCGTCTAC

       310         320         330         340         350         360
ACCCCCGCCA TGGAGAGTGT CTGCGGATAC TTCCACAGGT CCCACAACCG CAGCGAGGAG

       370         380         390         400         410         420
TTTCTCATTG CTGGAAAACT GCAGGATGGA CTCTTGCACA TCACTACCTG CAGTTTCGTG

       430
GCTCCCTGGA AC
```

In this second preferred sequence, an open reading frame exists from nucleotides 1 through 432. The first methionine of this reading frame is encoded by nucleotides by 49 through 51 and is the site of translation initiation. It should be noted that the amino acid sequence prescribed by nucleotides 49 through 114 is

not found in the mature metalloproteinase. It is believed that this sequence is the leader peptide of the human protein.

A third preferred portable DNA sequence has the nucleotide sequence:

```
        10          20          30          40          50          60
GGCCATCGCC GCAGATCCAG CGCCCAGAGA GACACCAGAG AACCCACCAT GGCCCCCTTT

        70          80          90          100         110         120
GACCCCTGGC TTCTGCATCC TGTTGTTGCT GTGGCTGATA GCCCCAGCAG GGCCTGCACC

        130         140         150         160         170         180
TGTGTCCCAC CCCACCCACA GACGGCCTTC TGCAATTCCG ACCTCGTCAT CAGGGCCAAG

        190         200         210         220         230         240
TTCGTGGGGA CACCAGAAGT CAACCAGACC ACCTTATACC AGCGTTATGA GATCAAGATG

        250         260         270         280         290         300
ACCAAGATGT ATAAAGGGTT CCAAGCCTTA GGGGATGCCG CTGACATCCG GTTCGTCTAC

        310         320         330         340         350         360
ACCCCCGCCA TGGAGAGTGT CTGCGGATAC TTCCACAGGT CCCACAACCG CAGCGAGGAG

        370         380         390         400         410         420
TTTCTCATTG CTGGAAAACT GCAGGATGGA CTCTTGCACA TCACTACCTG CAGTTTCGTG

        430         440         450         460         470         480
GCTCCCTGGA ACAGCCTGAG CTTAGCTCAG CGCCGGGGCT TCACCAAGAC CTACACTGTT

        490         500         510         520         530         540
GGCTGTGAGG AATGCACAGT GTTTCCCTGT TTATCCATCC CCTGCAAACT GCAGAGTGGC

        550         560         570         580         590         600
ACTCATTGCT TGTGGACGGA CCAGCTCCTC CAAGGCTCTG AAAAGGGCTT CCAGTCCCGT

        610         620         630         640         650         660
CACCTTGCCT GCCTGCCTCG GGAGCCAGGG CTGTGCACCT GGCAGTCCCT GCGGTCCCAG
```

670 680 690 700 710 720
ATAGCCTGAA TCCTGCCCGG AGTGGAAGCT GAAGCCTGCA CAGTGTCCAC CCTGTTCCCA

730 740 750 760 770 780
CTCCCATCTT TCTTCCGGAC AATGAAATAA AGAGTTACCA CCCAGCAAAA AAAAAAAGGA

This third sequence contains the 5' nontranslated region of the second preferred sequence and the 3' region of the first preferred sequence. It is envisioned that this third preferred sequence is capable of directing intracellular production of a metalloproteinase analogous to a mature human collagenase inhibitor in a microbial or mammalian expression system.

Currently, for expression of the instant metalloproteinase inhibitors in animal cells, the inventors most prefer a method which utilizes a fourth preferred DNA sequence. The coding strand of this sequence reads as follows:

10 20 30 40 50 60
GGCCATCGCC GCAGATCCAG CGCCCAGAGA GACACCAGAG AACCCACCAT GGCCCCCTTT

70 80 90 100 110 120
GAGCCCCTGG CTTCTGGCAT CCTGTTGTTG CTGTGGCTGA TAGCCCCCAG CAGGGCCTGC

130 140 150 160 170 180
ACCTGTGTCC CACCCCACCC ACAGACGGCC TTCTGCAATT CCGACCTCGT CATCAGGGCC

190 200 210 220 230 240
AAGTTCGTGG GGACACCAGA AGTCAACCAG ACCACCTTAT ACCAGCGTTA TGAGATCAAG

250 260 270 280 290 300
ATGACCAAGA TGTATAAAGG GTTCCAAGCC TTAGGGGATG CCGCTGACAT CCGGTTCGTC

310 320 330 340 350 360
TACACCCCCG CCATGGAGAG TGTCTGCGGA TACTTCCACA GGTCCCACAA CCGCAGCGAG

370 380 390 400 410 420
GAGTTTCTCA TTGCTGGAAA ACTGCAGGAT GGACTCTTGC ACATCACTAC CTGCAGTTTC

430 440 450 460 470 480
GTGGCTCCCT GGAACAGCCT GAGCTTAGCT CAGCGCCGGG GCTTCACCAA GACCTACACT

```
      490         500         510         520         530         540
GTTGGCTGTG AGGAATGCAC AGTGTTTCCC TGTTTATCCA TCCCCTGCAA ACTGCAGAGT

      550         560         570         580         590         600
GGCACTCATT GCTTGTGGAC GGACCAGCTC CTCCAAGGCT CTGAAAAGGG CTTCCAGTCC

      610         620         630         640         650         660
CGTCACCTTG CCTGCCTGCC TCGGGAGCCA GGGCTGTGCA CCTGGCAGTC CCTGCGGTCC

      670         680         690         700         710         720
CAGATAGCCT GAATCCTGCC CGGAGTGGAA GCTGAAGCCT GCACAGTGTC CACCCTGTTC

      730         740         750         760         770         780
CCACTCCCAT CTTTCTTCCG GACAATGAAA TAAAGAGTTA CCACCCAGCA AAAAAAAAAA
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It must be borne in mind in the practice of the present invention that the alteration of some amino acids in a protein sequence may not affect the fundamental properties of the protein. Therefore, it is also contemplated that other portable DNA sequences, both those capable of directing intracellular production of identical amino acid sequences and those capable of directing intracellular production of analogous amino acid sequences which also possess metalloproteinase inhibitor activity, are included within the ambit of the present invention.

It is contemplated that some of these analogous amino acid sequences will be substantially homologous to native human metalloproteinase inhibitors while other amino acid sequences, capable of functioning as metalloproteinase inhibitors, will not exhibit substantial homology to native inhibitors. By "substantial homology," as used herein, is meant a degree of homology to a native metalloproteinase inhibitor in excess of 50%, preferably in excess of 60%, preferably in excess of 80%. The percentage homology as discussed herein is calculated as the percentage of amino acid residues found in the smaller of the two sequences that align with identical amino acid residues in the sequence being compared when four gaps in a length of 100 amino acids may be introduced to assist in that alignment as set forth by Dayhoff, M.O. in Atlas of Protein Sequence and Structure Vol. 5, p. 124 (1972), National Biochemical Research Foundation, Washington, D.C., specifically incorporated herein by reference.

As noted above, the portable DNA sequences of the present invention may be synthetically created. It is believed that the means for synthetic creation of these polynucleotide sequences are generally known to one of ordinary skill in the art, particularly in light of the teachings contained herein. As an example of the current state of the art relating to polynucleotide synthesis, one is directed to Matteucci, M.D. and Caruthers, M.H., in J. Am. Chem. Soc. 103: 3185 (1981) and Beaucage, S.L. and Caruthers, M.H. in Tetrahedron Lett. 22: 1859 (1981), specifically incorporated herein by reference.

Additionally, the portable DNA sequence may be a fragment of a natural sequence, i.e., a fragment of a polynucleotide which occurred in nature and which has been isolated and purified for the first time by the present inventors. In one embodiment, the portable DNA sequence is a restriction fragment isolated from a cDNA library. In this preferred embodiment, the cDNA library is created from human skin fibroblasts.

In an alternative embodiment, the portable DNA sequence is isolated from a human genomic library. An example of such a library useful in this embodiment is set forth in Lawn et al. Cell 15: 1157-1174 (1978), specifically incorporated herein by reference.

As also noted above, the present invention relates to a series of vectors, each containing at least one of the portable DNA sequences described herein. It is contemplated that additional copies of the portable DNA sequence may be included in a single vector to increase a host microorganism's ability to produce large quantities of the desired metalloproteinase inhibitor.

In addition, the cloning vectors within the scope of the present invention may contain supplemental nucleotide sequences preceding or subsequent to the portable DNA sequence. These supplemental sequences are those that will not interfere with transcription of the portable DNA sequence and will, in some instances as set forth more fully hereinbelow, enhance transcription, translation, or the ability of the primary amino acid structure of the resultant metalloproteinase inhibitor to assume an active, tertiary form.

A preferred vector of the present invention is set forth in Figure 1. This vector, pUC9-F5/237P10, contains the preferred nucleotide sequence set forth above. Vector pUC9-F5/237P10 is present in the C600/pUC9-F5/237P10 cells on deposit in the American Type Culture Collection in Rockville, Maryland under Accession No. 53003.

A preferred nucleotide sequence encoding the metalloproteinase inhibitor is identified in Figure 1 as region A. Plasmid pUC9-F5/237P10 also contains supplemental nucleotide sequences preceding and subsequent to the preferred portable DNA sequence in region A. These supplemental sequences are identified as regions B and C, respectively.

In alternate preferred embodiments, either one or both of the preceding or subsequent supplemental sequences may be removed from the vector of Fig. 1 by treatment of the vector with restriction endonucleases appropriate for removal of the supplemental sequences. The supplemental sequence subsequent to the portable DNA sequence, identified in Fig. 1 as region C, may be removed by treatment of the vector with a suitable restriction endonuclease, preferably HgiAI followed by reconstruction of the 3' end of region A using synthetic oligonucleotides and ligation of the vector with T-4 DNA ligase. Deletion of the supplemental sequence preceding the portable DNA sequence, identified as region B in Fig. 1, would be specifically accomplished by the method set forth in Example 2.

In preferred embodiments, cloning vectors containing and capable of expressing the portable DNA sequence of the present invention contain various operational elements. These "operational elements," as discussed herein, include at least one promoter, at least one Shine-Dalgarno sequence, at least one terminator codon. Preferably, these "operational elements" also include at least one operator, at least one leader sequence, and for proteins to be exported from intracellular space, at least one regulator and any other DNA sequences necessary or preferred for appropriate transcription and subsequent translation of the vector DNA.

Additional embodiments of the present invention are envisioned as employing other known or currently undiscovered vectors which would contain one or more of the portable DNA sequences described herein. In particular, it is preferred that these vectors have some or all of the following characteristics: (1) possess a minimal number of host-organism sequences; (2) be stable in the desired host; (3) be capable of being present in a high copy number in the desired host; (4) possess a regulatable promoter; (5) have at least one DNA sequence coding for a selectable trait present on a portion of the plasmid separate from that where the portable DNA sequence will be inserted; and (6) be integrated into the vector.

The following, noninclusive, list of cloning vectors is believed to set forth vectors which can easily be altered to meet the above-criteria and are therefore preferred for use in the present invention. Such alterations are easily performed by those of ordinary skill in the art in light of the available literature and the teachings herein.

TABLE I

HOST	Vectors	Comments
E. coli	pUC8 pUC9 pBR322 pGW7 placI^{q} pDP8	Many selectable replicons have been characterized. Maniatis, T. et al. (1982), Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory.
BACILLUS B. subtilis B. amyloliquefaciens B. stearothermophilus	pUB110 pSA0501 pSA2100 pBD6 pBD8 pT127	Genetics and Biotechnology of Bacilli, Ganesan and Hoch, eds., 1984, Academic Press.

PSEUDOMONAS	RSF1010	Some vectors useful in
P. aeruginosa	Rms149	broad host range of gram-
P. putida	pKT209	negative bacteria including
	RK2	Xanthomonas and
		Agrobacterium.
	pSa727	
CLOSTRIDIUM	pJU12	Shuttle plasmids for E.
C. perfringens	pJU7	coli and C. perfringens
	pJU10	construction ref. Squires,
	pJU16	C. et al. (1984) Journal
	pJU13	Bacteriol. 159:465-471.
SACCHAROMYCES	YEp24	Botstein and Davis in
S. cerevisiae	YIp5	Molecular Biology of the
	YRp17	Yeast Saccharomyces,
		Strathern, Jones, and
		Broach, eds., 1982, Cold
		Spring Harbor Laboratory.

It is to be understood that additional cloning vectors may now exist or will be discovered which have the above-identified properties and are therefore suitable for use in the present invention. These vectors are also contemplated as being within the scope of the disclosed series of cloning vectors into which the portable DNA sequences may be introduced, along with any necessary operational elements, and which altered vector is then included within the scope of the present invention and would be capable of being used in the recombinant-DNA method set forth more fully below.

In addition to the above list, an E. coli vector system, as set forth in Example 2, is preferred in one embodiment as a cloning vector. Moreover, several vector plasmids which autonomously replicate in a broad range of Gram Negative bacteria are preferred for use as cloning vehicles in hosts of the genera Pseudomonas. These are described by Tait, R.C., Close, T.J., Lundquist, R.C., Hagiya, M., Rodriguez, R.L., and Kado, C.I. in Biotechnology, May, 1983, pp. 269-275; Panopoulos, N.J. in

Genetic Engineering in the Plant Sciences, Praeger Publishers, New York, New York, pp. 163-185, (1981); and Sakaguchi, K. in Current Topic in Microbiology and Immunology 96:31-45, (1982), each of which is specifically incorporated herein by reference.

One particularly preferred construction employs the plasmid RSF1010 and derivatives thereof as described by Bagdasarian, M., Bagdasarian, M.M., Coleman, S., and Timmis, K.N. in Plasmids of Medical Environmental and Commercial Importance, Timmis, K.N. and Puhler, A. eds., Elsevier/North Holland Biomedical Press, (1979), specifically incorporated herein by reference. The advantages of RSF1010 are that it is relatively small, high copy number plasmid which is readily transformed into and stably maintained in both E. coli and Pseudomonas species. In this system, it is preferred to use the Tac expression system as described for Escherichia, since it appears that the E. coli trp promoter is readily recognized by Pseudomonas RNA polymerase as set forth by Sakaguchi, K. in Current Topics in Microbiology and Immunology 96:31-45 (1982) and Gray, G.L., McKeown, K.A., Jones, A.J.S., Seeburg, P.H., and Heyneker, H.L. in Biotechnology Feb. 1984, pp. 161-165, both of which are specifically incorporated herein by reference. Transcriptional activity may be further maximized by requiring the exchange of the promoter with, e.g., an E. coli or P. aeruginosa trp promoter.

In a preferred embodiment, P. aeruginosa is transformed with vectors directing the synthesis of the metalloproteinase inhibitor as either an intracellular product or as a product coupled to leader sequences that will effect its processing and export from the cell. In this embodiment, these leader sequences are preferably selected from the group consisting of beta-lactamase, OmpA protein, the naturally occurring human signal peptide, and that of carboxypeptidase G2 from Pseudomonas. Translation may be coupled to translation initiation for any of the E. coli proteins as described in Example 2, as well as to initiation sites for any of the highly expressed proteins of the host to cause intracellular expression of the metalloproteinase inhibitor.

In those cases where restriction minus strains of a host Pseudomonas species are not available, transformation efficiency with plasmid constructs isolated from E. coli are poor. Therefore, passage of the Pseudomonas cloning vector through an r- m+ strain of another species prior to transformation of the desired host, as set forth in Bagdasarian, M., et al., Plasmids of Medical, Environmental and Commercial Importance, pp. 411-422, Timmis and Puhler eds., Elsevier/North Holland Biomedical Press (1979), specifically incorporated herein by reference, is desired.

Furthermore, a preferred expression system in hosts of the genera Bacillus involves using plasmid pUB110 as the cloning vehicle. As in other host vector systems, it is possible in Bacillus to express the metalloproteinase inhibitors of the present invention as either an intracellular or a secreted protein. The present embodiments include both systems. Shuttle vectors that replicate in both Bacillus and E. coli are available for constructing and testing various genes as described by Dubnau, D., Gryczan, T., Contente, S., and Shivakumar, A.G. in Genetic Engineering, Vol. 2, Setlow and Hollander eds., Plenum Press, New York, New York, pp. 115-131, (1980), specifically incorporated herein by reference. For the expression and secretion of metalloproteinase inhibitors from B. subtilis, the signal sequence of alpha-amylase is preferably coupled to the coding region for the metalloproteinase inhibitor. For synthesis of intracellular metalloproteinase inhibitor, the portable DNA sequence will be translationally coupled to the ribosome binding site of the alpha-amylase leader sequence.

Transcription of either of these constructs is preferably directed by the alpha-amylase promoter or a derivative thereof. This derivative contains the RNA polymerase recognition sequence of the native alpha-amylase promoter but incorporates the lac operator region as well. Similar hybrid promoters constructed from the penicillinase gene promoter and the lac operator have been shown to function in Bacillus hosts in a regulatable fashion as set forth by Yansura, D.G. and Henner in Genetics and Biotechnology of Bacilli, Ganesan, A.T. and Hoch,

J.A., eds., Academic Press, pp. 249-263, (1984), specifically incorporated by reference. The lacI gene of $lacI^q$ would also be included to effect regulation.

One preferred construction for expression in Clostridium is in plasmid pJU12 described by Squires, C. H. et al in J. Bacteriol. 159:465-471 (1984), specifically incorporated herein by reference, transformed into C. perfringens by the method of Heefner, D. L. et al. as described in J. Bacteriol. 159:460-464 (1984), specifically incorporated herein by reference. Transcription is directed by the promoter of the tetracycline resistance gene. Translation is coupled to the Shine-Dalgarno sequences of this same tet^r gene in a manner strictly analogous to the procedures outlined above for vectors suitable for use in other hosts.

Maintenance of foreign DNA introduced into yeast can be effected in several ways (Botstein, D., and Davis, R. W., in The Molecular Biology of the Yeast Saccharomyces, Cold Spring Harbor Laboratory, Strathern, Jones and Broach, eds., pp. 607-636 (1982). One preferred expression system for use with host organisms of the genus Saccharomyces harbors the anticollagenase gene on the 2 micron plasmid. The advantages of the 2 micron circle include relatively high copy number and stability when introduced into cir° strains. These vectors preferably incorporate the replication origin and at least one antibiotic resistance marker from pBR322 to allow replication and selection in E. coli. In addition, the plasmid will preferably have 2 micron sequences and the yeast LEU2 gene to serve the same purposes in LEU2 mutants of yeast.

The regulatable promoter from the yeast GAL1 gene will preferably be adapted to direct transcription of the portable DNA sequence gene. Translation of the portable DNA sequence in yeast will be coupled to the leader sequence that directs the secretion of yeast alpha-factor. This will cause formation of a fusion protein which will be processed in yeast and result in secretion of a metalloproteinase inhibitor. Alternatively, a methionyl-metalloproteinase inhibitor will be translated for inclusion within the cell.

It is anticipated that translation of mRNA coding for the metalloproteinase inhibitor in yeast will be more efficient with the preferred codon usage of yeast than with the sequence present in pUC8-Fic, as identified in Example 2, which has been tailored to the prokaryotic bias. For this reason, the portion of the 5' end of the portable DNA sequence beginning at the Tth111I site is preferably resynthesized. The new sequence favors the codons most frequently used in yeast. This new sequence preferably has the following nucleotide sequence:

```
              HgiAI
5'   GAT CCG TGC ACT TGT GTT CCA CCA CAC
          GC ACG TGA ACA CAA GGT GGT GTG

     CCA CAA ACT GCT TTC TGT AAC TCT GAC C
     GGT GTT TGA CGA AAG ACA TTG AGA CTG GA    3'
```

As will be seen from an examination of the individual cloning vectors and systems contained on the above list and description, various operational elements may be present in each of the preferred vectors of the present invention. It is contemplated any additional operational elements which may be required may be added to these vectors using methods known to those of ordinary skill in the art, particularly in light of the teachings herein.

In practice, it is possible to construct each of these vectors in a way that allows them to be easily isolated, assembled, and interchanged. This facilitates assembly of numerous functional genes from combinations of these elements and the coding region of the metalloproteinase inhibitor. Further, many of these elements will be applicable in more than one host.

At least one origin of replication recognized by the contemplated host microorganism, along with at least one selectable marker and at least one promoter sequence capable of initiating transcription of the portable DNA sequence are contemplated as being included in these vectors. It is additionally contemplated that the vectors, in certain preferred embodiments, will contain DNA sequences capable of functioning as regulators ("operators"), and other DNA sequences capable of coding for

regulator proteins. In preferred vectors of this series, the vectors additionally contain ribosome binding sites, transcription terminators and leader sequences.

These regulators, in one embodiment, will serve to prevent expression of the portable DNA sequence in the presence of certain environmental conditions and, in the presence of other environmental conditions, allow transcription and subsequent expression of the protein coded for by the portable DNA sequence. In particular, it is preferred that regulatory segments be inserted into the vector such that expression of the portable DNA sequence will not occur in the absence of, for example, isopropylthio-β-d-galactoside. In this situation, the transformed microorganisms containing the portable DNA may be grown to a desired density prior to initiation of the expression of the metalloproteinase inhibitor. In this embodiment, expression of the desired protease inhibitor is induced by addition of a substance to the microbial environment capable of causing expression of the DNA sequence after the desired density has been achieved.

Additionally, it is preferred that an appropriate secretory leader sequence be present, either in the vector or at the 5' end of the portable DNA sequence, the leader sequence being in a position which allows the leader sequence to be immediately adjacent to the initial portion of the nucleotide sequence capable of directing expression of the protease inhibitor without any intervening transcription or translation termination signals. The presence of the leader sequence is desired in part for one or more of the following reasons: 1) the presence of the leader sequence may facilitate host processing of the initial product to the mature recombinant metalloproteinase inhibitor; 2) the presence of the leader sequence may facilitate purification of the recombinant metalloproteinase inhibitors, through directing the metalloproteinase inhibitor out of the cell cytoplasm; 3) the presence of the leader sequence may affect the ability of the recombinant metalloproteinase inhibitor to fold to its active structure through directing the metalloproteinase inhibitor out of the cell cytoplasm.

In particular, the leader sequence may direct cleavage of the initial translation product by a leader peptidase to remove the leader sequence and leave a polypeptide with the amino acid sequence which has the potential of metalloproteinase inhibitory activity. In some species of host microorganisms, the presence of the appropriate leader sequence will allow transport of the completed protein into the periplasmic space, as in the case of E. coli. In the case of certain yeasts and strains of Bacillus and Pseudomonas, the appropriate leader sequence will allow transport of the protein through the cell membrane and into the extracellular medium. In this situation, the protein may be purified from extracellular protein.

Thirdly, in the case of some of the metalloproteinase inhibitors prepared by the present invention, the presence of the leader sequence may be necessary to locate the completed protein in an environment where it may fold to assume its active structure, which structure possesses the appropriate metalloproteinase activity.

Additional operational elements include, but are not limited to, ribosome-binding sites and other DNA sequences necessary for microbial expression of foreign proteins. The operational elements as discussed herein can be routinely selected by those of ordinary skill in the art in light of prior literature and the teachings contained herein. General examples of these operational elements are set forth in B. Lewin, Genes, Wiley & Sons, New York (1983), which is specifically incorporated herein by reference. Various examples of suitable operational elements may be found on the vectors discussed above and may be elucidated through review of the publications discussing the basic characteristics of the aforementioned vectors.

In one preferred embodiment of the present invention, an additional DNA sequence is located immediately preceding the portable DNA sequence which codes for the metalloproteinase inhibitor. The additional DNA sequence is capable of functioning as a translational coupler, i.e., it is a DNA sequence that encodes an RNA which serves to position ribosomes immediately adjacent to the ribosome binding site of the metalloproteinase inhibitor RNA with which it is contiguous.

Upon synthesis and/or isolation of all necessary and desired component parts of the above-discussed cloning vectors, the vectors are assembled by methods generally known to those of ordinary skill in the art. Assembly of such vectors is believed to be within the duties and tasks performed by those with ordinary skill in the art and, as such, is capable of being performed without undue experimentation. For example, similar DNA sequences have been ligated into appropriate cloning vectors, as set forth in Schoner et al., Proceedings of the National Academy of Sciences U.S.A., 81:5403-5407 (1984), which is specifically incorporated herein by reference.

In construction of the cloning vectors of the present invention, it should additionally be noted that multiple copies of the portable DNA sequence and its attendant operational elements may be inserted into each vector. In such an embodiment, the host organism would produce greater amounts per vector of the desired metalloproteinase inhibitor. The number of multiple copies of the DNA sequence which may be inserted into the vector is limited only by the ability of the resultant vector, due to its size, to be transferred into and replicated and transcribed in an appropriate host microorganism.

Additionally, it is preferred that the cloning vector contain a selectable marker, such as a drug resistance marker or other marker which causes expression of a selectable trait by the host microorganism. In a particularly preferred embodiment of the present invention, the gene for ampicillin resistance is included in vector pUC9-F5/237P10.

Such a drug resistance or other selectable marker is intended in part to facilitate in the selection of transformants. Additionally, the presence of such a selectable marker on the cloning vector may be of use in keeping contaminating microorganisms from multiplying in the culture medium. In this embodiment, such a pure culture of the transformed host microorganisms would be obtained by culturing the microorganisms under conditions which require the induced phenotype for survival.

It is noted that, in preferred embodiment, it is also desirable to reconstruct the 3' end of the coding region to allow

assembly with 3' non-translated sequences. Included among these non-translated sequences are those which stabilize the mRNA or enhance its transcription and those that provide strong transcriptional termination signals which may stabilize the vector as they are identified by Gentz, R., Langner, A., Chang, A.C.Y., Cohen, S.H., and Bujard, H. in Proc. Natl. Acad. Sci. USA 78:4936-4940 (1981), specifically incorporated herein by reference.

This invention also relates to a recombinant-DNA method for the production of metallproteinase inhibitors. Generally, this method includes:

(a) preparation of a portable DNA sequence capable of directing a host microorganism to produce a protein having metalloproteinase inhibitor activity;

(b) cloning the portable DNA sequence into a vector capable of being transferred into and replicating in a host microorganism, such vector containing operational elements for the portable DNA sequence;

(c) transferring the vector containing the portable DNA sequence and operational elements into a host microorganism capable of expressing the metalloproteinase inhibitor protein;

(d) culturing the host microorganism under conditions appropriate for amplification of the vector and expression of the inhibitor; and

(e) in either order:

(i) harvesting the inhibitor; and

(ii) causing the inhibitor to assume an active, tertiary structure whereby it possesses metalloproteinase inhibitor activity.

In this method, the portable DNA sequences are those synthetic or naturally-occurring polynucleotides described above.

In a preferred embodiment of the present method, the portable DNA sequence has the nucleotide sequence as follows:

```
        10          20          30          40          50          60
GTTGTTGCTG TGGCTGATAG CCCCAGCAGG GCCTGCACCT GTGTCCCACC CCACCCACAG

        70          80          90         100         110         120
ACGGCCTTCT GCAATTCCGA CCTCGTCATC AGGGCCAAGT TCGTGGGGAC ACCAGAAGTC

       130         140         150         160         170         180
AACCAGACCA CCTTATACCA GCGTTATGAG ATCAAGATGA CCAAGATGTA TAAAGGGTTC

       190         200         210         220         230         240
CAAGCCTTAG GGGATGCCGC TGACATCCGG TTCGTCTACA CCCCCGCCAT GGAGAGTGTC

       250         260         270         280         290         300
TGCGGATACT TCCACAGGTC CCACAACCGC AGCGAGGAGT TTCTCATTGC TGGAAAACTG

       310         320         330         340         350         360
CAGGATGGAC TCTTGCACAT CACTACCTGC AGTTTCGTGG CTCCCTGGAA CAGCCTGAGC

       370         380         390         400         410         420
TTAGCTCAGC GCCGGGGCTT CACCAAGACC TACACTGTTG GCTGTGAGGA ATGCACAGTG

       430         440         450         460         470         480
TTTCCCTGTT TATCCATCCC CTGCAAACTG CAGAGTGGCA CTCATTGCTT GTGGACGGAC

       490         500         510         520         530         540
CAGCTCCTCC AAGGCTCTGA AAAGGGCTTC CAGTCCCGTC ACCTTGCCTG CCTGCCTCGG

       550         560         570         580         590         600
GAGCCAGGGC TGTGCACCTG GCAGTCCCTG CGGTCCCAGA TAGCCTGAAT CCTGCCCGGA

       610         620         630         640         650         660
GTGGAAGCTG AAGCCTGCAC AGTGTCCACC CTGTTCCCAC TCCCATCTTT CTTCCGGACA

       670         680         690         700
ATGAAATAAA GAGTTACCAC CCAGCAAAAA AAAAAAGGAA TTC
```

The vectors contemplated as being useful in the present method are those described above. In a preferred embodiment, the cloning vector pUC9-F5/237P10 is used in the disclosed method.

The vector thus obtained is then transferred into the appropriate host microorganism. It is believed that any microorganism having the ability to take up exogenous DNA and express those genes and attendant operational elements may be chosen. It is preferred that the host microorganism be an anaerobe, facultative anaerobe or aerobe. Particular hosts which may be preferable for use in this method include yeasts and bacteria. Specific yeasts include those of the genus Saccharomyces, and especially Saccharomyces cerevisiae.

Specific bacteria include those of the genera Bacillus and Escherichia and Pseudomonas. Various other preferred hosts are set forth in Table I, supra. In other, alternatively preferred embodiments of the present invention, Bacillus subtilis, Escherichia coli or Pseudomonas aeruginosa are selected as the host microorganisms.

After a host organism has been chosen, the vector is transferred into the host organism using methods generally known by those of ordinary skill in the art. Examples of such methods may be found in Advanced Bacterial Genetics by R. W. Davis et al., Cold Spring Harbor Press, Cold Spring Harbor, New York, (1980), which is specifically incorporated herein by reference. It is preferred, in one embodiment, that the transformation occur at low temperatures, as temperature regulation is contemplated as a means of regulating gene expression through the use of operational elements as set forth above. In another embodiment, if osmolar regulators have been inserted into the vector, regulation of the salt concentrations during the transformation would be required to insure appropriate control of the synthetic genes.

If it is contemplated that the recombinant metalloproteinase inhibitors will ultimately be expressed in yeast, it is preferred that the cloning vector first be transferred into Escherichia coli, where the vector would be allowed to replicate and from which the vector would be obtained and purified after amplification. The vector would then be transferred into the yeast for ultimate expression of the metalloproteinase inhibitor.

The host microorganisms are cultured under conditions appropriate for the expression of the metalloproteinase inhibitor. These conditions are generally specific for the host organism, and are readily determined by one of ordinary skill in the art, in light of the published literature regarding the growth conditions for such organisms, for example Bergey's Manual of Determinative Bacteriology, 8th Ed., Williams & Wilkins Company, Baltimore, Maryland, which is specifically incorporated herein by reference.

Any conditions necessary for the regulation of the expression of the DNA sequence, dependent upon any operational elements inserted into or present in the vector, would be in effect at the transformation and culturing stages. In one embodiment, the cells are grown to a high density in the presence of appropriate regulatory conditions which inhibit the expression of the DNA sequence. When optimal cell density is approached, the environmental conditions are altered to those appropriate for expression of the portable DNA sequence. It is thus contemplated that the production of the metalloproteinase inhibitor will occur in a time span subsequent to the growth of the host cells to near optimal density, and that the resultant metalloproteinase inhibitor will be harvested at some time after the regulatory conditions necessary for its expression were induced.

In a preferred embodiment of the present invention, the recombinant metalloproteinase inhibitor is purified subsequent to harvesting and prior to assumption of its active structure. This embodiment is preferred as the inventors believe that recovery of a high yield of re-folded protein is facilitated if the protein is first purified. However, in one preferred, alternate embodiment, the metalloproteinase inhibitor may be allowed re-fold to assume its active structure prior to purification. In yet another preferred, alternate embodiment, the metalloproteinase inhibitor is caused to assume its re-folded, active state upon recovery from the culturing medium.

In certain circumstances, the metalloproteinase inhibitor will assume its proper, active structure upon expression in the host microorganism and transport of the protein through the

cell wall or membrane or into the periplasmic space. This will generally occur if DNA coding for an appropriate leader sequence has been linked to the DNA coding for the recombinant protein. The preferred metalloprotienase inhibitors of the present invention will assume their mature, active form upon translocation out of the inner cell membrane. The structures of numerous signal peptides have been published, for example by Marion E.E. Watson in Nuc. Acid Res. 12:515-5164, 1984, specifically incorporated herein by reference. It is intended that these leader sequences, together with portable DNA, will direct intracellular production of a fusion protein which will be transported through the cell membrane and will have the leader sequence portion cleaved upon release from the cell.

In a preferred embodiment, the signal peptide of the E.coli OmpA protein is used as a leader sequence and is located in a position contiguous with the portable DNA sequence coding for the metalloproteinese inhibitor structure.

Additionally preferred leader sequences include those of beta-lactamase, carboxypeptidase G2 and the human signal protein. These and other leader sequences are described.

If the metalloproteinase inhibitor does not assume its proper, active structure, any disulfide bonds which have formed and/or any noncovalent interactions which have occurred will first be disrupted by denaturing and reducing agents, for example, guanidinium chloride and β-mercaptoethanol, before the metalloproteinase inhibitor is allowed to assume its active structure following dilution and oxidation of these agents under controlled conditions.

The transcription terminators contemplated herein serve to stabilize the vector. In particular, those sequences as described by Gentz et al., in Proc. Natl. Acad. Sci. USA 78: 4936-4940 (1981), specifically incorporated herein by reference, are contemplated for use in the present invention.

It is to be understood that application of the teachings of the present invention to a specific problem or environment will be within the capabilities of one having ordinary skill in the art in light of the teachings contained herein. Examples

of the products of the present invention and representative processes for their isolation and manufacture appear in the following examples.

EXAMPLES

EXAMPLE 1

Preparation of Poly(A^+) RNA from HEF-SA Fibroblasts

HEF-SA cells were grown to near confluence in 75 cm^2 T-flasks. Cells were washed twice in Dulbecco's phosphate buffered saline solution and harvested by the addition of 2 ml of 10 mM Tris, pH 7.5 containing 1% w/v SDS (obtained from BDH chemicals, Ltd., Poole, England), 5 mM EDTA and 20 ug/ml protease K (obtained from Boehringer Mannheim Biochemicals, Indianapolis, Indiana). Each flask was subsequently washed with an additional milliliter of this same solution.

The pooled aliquots from the cell harvest were made to 70 ug/ml in protease K and incubated at 40°C for 45 minutes. The proteolyzed solution was brought to a NaCl concentration of 150 mM by the addition of 5 M stock and subsequently extracted with an equal volume of phenol:chloroform 1:1. The aqueous phase was reextracted with an equal volume of chloroform. Two volumes of ethanol were added to the aqueous phase and incubated overnight at -20°C. The precipitated nucleic acids were recovered by centrifugation at 17,500 xg for 10 minutes in a Beckman J2-21 centrifuge, Beckman Instruments, Palo Alto, California, and were redissolved in 25 ml of 0.1% w/v SDS. This solution was again extracted with an equal volume of chloroform. The aqueous phase was added to two volumes of cold ethanol and kept at -20°C for 2 hours. The precipitate was collected by centrifugation at 10,000 xg for 15 minutes and redissolved in 10 ml of 1 mM Tris, 0.5 mM EDTA, 0.1% SDS, pH 7.5. RNA was precipitated from this solution by the addition of 10 ml of 4 M LiCl, 20 mM NaoAc, pH 5.0 and incubated at -20°C for 18 hours. The precipitate was again recovered by centrifugation and washed twice with 2 M LiCl before redissolving in 1 mM Tris, 0.5 mM EDTA, 0.1% SDS, pH 7.5. This solution was stored at -70°C.

Chromatography on Oligo dT Cellulose

Total cellular RNA prepared as above was ethanol precipitated and redissolved in 0.5 M NaCl. Five ml of RNA at 0.45 mg/ml were applied to a 1 ml column of washed type VII oligo dT cellulose (obtained from PL Biochemicals, Milwaukee, Wisconsin). The column was then washed with 10 ml of 0.5 M NaCl and eluted with 2.0 ml of sterile H_2O. The eluted poly(A^+) fraction of RNA was ethanol precipitated and dissolved to give a 1 mg/ml solution in 1 mM Tris, 0.1 mM EDTA, pH 8.0. This was stored at -70°C.

cDNA Synthesis

Poly(A^+) RNA was primed with oligo dT (obtained from PL Biochemicals, Milwaukee, Wisconsin) to serve as a template for cDNA synthesis by AMV reverse transcriptase (obtained from Life Sciences, Inc., St. Petersburg, Florida). Following the synthesis reaction, the RNA was hydrolyzed by the addition of 0.1 volume of 3 N NaOH and incubated at 67°C for 10 minutes. The solution was then neutralized and the cDNA purified by gel filtration chromatography on biogel A 1.5 (obtained from BioRad Laboratories, Richmond, California) in a 0.7x25 cm column in a 10 mM Tris, 5 mM EDTA, and 1% SDS, pH 7.5 solution. Fractions containing cDNA were pooled and concentrated by ethanol precipitation. The cDNA was dG tailed and purified by gel filtration using the procedure set forth above. Second strand synthesis was primed with oligo dC and polymerized in an initial reaction with the large (Klenow) fragment of DNA polymerase (obtained from Boehringer Mannheim). Following second strand synthesis, E. coli DNA polymerase I (obtained from Boehringer Mannheim) was added and incubation continued to form blunt ends. The double stranded cDNA was again purified by chromatography. EcoRI restriction sites within the cDNAs were modified by the action of EcoRI methylase, obtained from New England Biolabs, Beverly, Massachusetts. The cDNA was again purified and ligated to synthetic EcoRI linkers. Finally, the ends were then trimmed with the endonuclease and the cDNA purified by gel filtration. This DNA was ligated into a unique EcoRI site in lambda gt10 DNA packaged in vitro and used to infect E. coli strain hflA according to the method set forth by Huynh, T.V., Young, R.A., and Davis, R.W., in

DNA Cloning Techniques, A Practical Approach (ed. Glover, D.M.) (IRL Press Oxford), in press, specifically incorporated herein by reference. Approximately 25,000 recombinants were amplified in this manner.

Screening

Recombinant-phage-containing sequences of interest were selected by their preferential hybridization to synthetic oligonucleotides encoding portions of the primary structure of the desired metalloproteinase inhibitor, hereinafter referred to as FIBAC. These portions of the protein sequence correspond in part to those set forth in the published literature by Stricklin, G.P. and Welgus, H.G., J. Biol. Chem. 258: 12252-12258 (1983), specifically incorporated herein by reference. Recombinant phage were used to infect E. coli strain hflA and plated at a density of approximately $2x10^3$ pfu/150 mm petri dish. Phage were blotted onto nitrocellulose filters (BA85, Schleicher & Schuell Inc., Keene, New Hampshire), and DNA was denatured and fixed essentially as described by Benton and Davis in Science 196:180-182 (1979) specifically incorporated herein by reference.

Using that procedure, the filters were treated sequentially for 10-15 minutes each in 0.5 M NaCl, then 1.0 M Tris, 1.5 M NaCl pH 8.0, and finally submerged in 2x SSPE. (2x SSPE is 0.36 M NaCl, 20 mM NaH_2PO_4, 2 mM EDTA pH 7.4). Filters were blotted dry and baked 75°-80° for 3-4 hours. Duplicate filters were made of each plate. Filters were prehybridized for 1-3 hours at 37° in 5x SSPE containing 0.1x SET, 0.15% NaPPi, and 1x Denhardts solutions. Filters were then hybridized for 72 hours at 37° in this same solution containing $5x10^5$ cpm/ml of 5' end-labeled 51-mer oligonucleotide specific activity approximately 10^6 cpm/pmole). Following hybridization, filters were washed six times in 5x SSPE containing 0.1x SET and 0.05% sodium pyrophosphate at 37°, then three times in 2x SSPE at 21°. These were then blotted dry and autoradiographed on Kodak XAR-5 film at -70° with a Kodak lightening-plus intensifying screen. Signals clearly visible from duplicate filters were used to pick phage for plaque purification. Filter preparations and hybridization procedures for plaque purification steps were the same as above.

The washing procedure was simplified to 6 changes of 2x SSPE at 37°. Six isolates purified by repetitive plating were then arranged on a single lawn of E. coli strain C600 for testing with subsequent probes.

Preferential hybridization of the 17-mer to each of the isolates (as opposed to control plaques) was observed under a condition identical to that used for plaque purification. Probe C was used in a similar test, except that the SSPE concentration during hybridization was reduced to 4x. Again, each of the isolates demonstrated stronger hybridization to the probe than did control plaques.

Phage Purification and cDNA Characterization

Quantities of each of the six isolated phage were made by the plate stock technique and purified by serial CsCl block gradient centrifugation. DNA was extracted from these by dialysis against 50% formamide as described by Davis, R.W., Botstein, D., Roth, J.R., in A Manual for Genetic Engineering: Advanced Bacterial Genetics, 1980, Cold Spring Harbor Laboratory, specifically incorporated herein by reference. DNA from each of the isolates was digested with EcoRI and the products were analyzed by agarose gel electrophoresis. The insert from one of the larger clones, lambda FIBAC 5, was found to lack internal sites for SalI, HindIII, BamHI, and EcoRI. The cDNA insert was released from lambda FIBAC 5 DNA and the lambda arms digested by co-digesting with these four enzymes. The fragments were then ethanol-precipitated and ligated into the EcoRI site of plasmid pUC9 without further purification. These plasmids were then used to transform E. coli strain JM83. Transformants were selected on ampicillin containing plates. Plasmids from several transformants were purified and characterized on the basis of the EcoRI digestion products. One was selected which had an insert co-migrating with the insert from lambda FIBAC 5 on agarose gel electrophoresis. This plasmid has been named pUC9-F5/237P10.

Mapping and Subcloning

The insert in pUC9-F5/237P10 was mapped with respect to internal PstI sites. Double digests with EcoRI and Pst demonstrated three internal PstI recognition sites. The entire insert